In search of biomarkers for leprosy diagnosis : in silico identification, screening & field application

Aboma, K.B.

Chapter 2

Post-genomic screening of Mycobacterium leprae proteins for immunogenicity and specificity

2.1 Immunogenicity of *Mycobacterium leprae* unique antigens in leprosy endemic populations in Asia and Africa

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Abstract

Ongoing transmission of leprosy is evident from the stable disease incidence in high burden areas. Tools for early detection of *Mycobacterium leprae* (*M. leprae*) infection, particularly in sub-clinically infected individuals, are urgently required to reduce transmission. Following the sequencing of the *M. leprae* genome, many *M. leprae*-unique candidate proteins have been identified, several of which have been tested for induction of *M. leprae* specific T cell responses in different leprosy endemic areas.

In this study, 21 *M. leprae*-unique proteins and 10 peptide pools covering the complete sequence of five *M. leprae*-unique proteins (ML0576, ML1989, ML1990, ML2283, and ML2567) were evaluated in 160 individuals in Nepal and Ethiopia. These included: tuberculoid and borderline tuberculoid (TT/BT), borderline borderline and borderline lepromatous (BB/BL) leprosy patients; healthy household contacts (HHC); tuberculosis (TB) patients and endemic controls (EC). Immunogenicity of the proteins was determined by IFN-γ secretion via stimulation of PBMC in 6 days lymphocyte stimulation tests (LST) or in whole blood assays (WBA).

In LST, BB/BL patients (40%) responded to ML0573 and ML1601 whereas ML1604 was most immunogenic in TT/BT (35%) and HHC (36%). Additionally, significant numbers of EC displayed IFN-γ production in response to ML0573 (54%), ML1601 (50%) and ML1604 (54%). TB patients on the other hand, hardly responded to any of the proteins except for ML1989. Comparison of IFN-γ responses to ML0121, ML0141 and ML0188 for TT/BT patients showed specific increase in diluted 6 days WBA compared to the undiluted 24 hours WBA, whereas EC showed a reduced response in the diluted WBA, which may indicate detection of disease-specific responses in the 6 days WBA.

In summary, identification of multiple *M. leprae* proteins inducing *M. leprae*-specific T cell responses in groups at high risk of developing leprosy may contribute to improve early detection for *M. leprae* infection.

Introduction

The introduction of multidrug treatment (MDT) recommended by WHO in 1981 aimed at eradication of leprosy by 2000 [17]. The shortened treatment period, 6 months and 1 year for paucibacillary (PB) (which includes TT/BT) and multibacillary (MB) (which includes BB/BL/LL) patients respectively, was one of the major steps forward by WHO which improved adherence and contributed to the dramatic reduction of leprosy prevalence globally. According to the WHO, eradication of leprosy as a public health problem (defined by less than one case per 10,000 people) has been achieved globally. High endemic zones, however, still subsist. The percentages of children among new cases of leprosy, which reflect a country's endemic level, ranged from 0.6 % in Argentina to 30.3 % in Papua New Guinea Moreover, the annual new case detection remains stable in many endemic countries [1]. This steady new case detection and the significant number of patients reporting with grade 2 disabilities, visible and irreversible, urge the need for strong efforts in discovering tools for early detection of *M. leprae* infection [1;5;14;19].

The disabilities and deformities in leprosy affected people along with wrong perception of the disease itself in most societies are the main reasons for stigma and discrimination. Despite the fact that the patients are cured from the disease with MDT, most people left out to live with unbearable social and psychological damage [3;12;13;16;21]. Hence, WHO has developed a new strategy for the next 5 years to mainly work on reducing disabilities, stigma and discrimination through provision of high quality patient care and early detection of new cases is obviously critical for the success of this effort [1]. However, do the available diagnostic tools sufficiently help in detecting leprosy infections at early (pre-clinical) stages?

Multibacillary leprosy patients (MB) are generally known to be main sources of *M. leprae* transmission. Close contacts of these patients who themselves are at high risk of developing the disease are believed to be asymptomatic sources of infection. However, the available diagnostic tools are not fully capable of detecting infections in these groups [5;8-9;11;20]. The clinical signs and symptoms, skin lesions consistent with leprosy having definite sensory loss, with or without thickened nerves and positive skin smears [18] are generally helpful to diagnose already advanced leprosy cases only. The anti-PGL-I (phenolglycolipid) IgM antibody measurement which mainly detects MB cases and which can also be positive for asymptomatic HHC, has been used for epidemiological and other related studies but is not applicable for routine laboratory diagnosis [15]. The lepromin test, on the other hand, measures the potential of an individual in building up granulomatous response to the mixture of *M. leprae* antigens of which lepromin is composed. An individual can develop a positive response to the test without ever having had any exposure to *M. leprae* [19]. This non-specific cellular response is caused by the fact that lepromin shares many proteins with other bacteria thereby inducing cross reactivity.

As in the development of TB diagnostics, the current high-tech era in genetics and bio-informatics has also contributed to the recent progress in the area of searching for potential proteins and peptides specific to *M. leprae* infection as the whole genome of the bacterium is sequenced [6;8-9;11;20]. Recent immunological studies in individuals from endemic countries revealed potential *M. leprae* proteins and peptides that induce T-cell responses. In Brazilian patients, ML1989, ML1990, ML2283 and ML2567 induced IFN-γ responses specific to *M. leprae* exposure [8]. However, further evaluation of these proteins combined with another potential specific protein ML2346 [6] and several *M. leprae* peptides [20] in five leprosy endemic countries (Brazil, Pakistan, Bangladesh, Ethiopia and Nepal) showed

significant responses in patients, but also in endemic controls (EC) as well as in healthy household controls [9]. This significant response in endemic controls raised the question whether reactivity to these proteins depicts exposure to or infection with *M. leprae* and whether the prevalence of leprosy in a region influences the responses induced in EC by *M. leprae* antigens. Thus this indicates the demand for continuous search of new *M. leprae* proteins and peptides that can induce T cell responses specific for *M. leprae* [10;11].

T-cell responses induced by mycobacterial peptides are more specific than those in response to mycobacterial proteins although the level of response in some assays is low [9;20]. The diversity of HLA in the human population will however require screening of large sets of *M. leprae* peptides in different leprosy endemic countries for possible wider application.

In this study, 21 recombinant *M. leprae* proteins, identified previously using a post genomic approach [8], were evaluated in leprosy patients, healthy household contacts, endemic controls and TB patients (completed intensive phase) from two leprosy endemic countries, Nepal and Ethiopia. In addition, pools of peptides covering five specific *M. leprae* proteins (ML0576, ML1989, ML2283, ML2346 and ML2567) previously tested in a Brazilian population [9;11] were further evaluated in the context of these Asian and African populations.

Materials and Methods

General procedure of the study. In this study, Armauer Hansen Research Institute, Addis Ababa, Ethiopia and the Mycobacterial Research Laboratory, Anandaban Hospital, Anandaban, Nepal, both located in leprosy endemic areas were involved in recruitment of patients, endemic controls and healthy household contacts. In both sites, identical standard operating procedures (SOPs) were applied and identical reagents were used to ensure reproducibility of data.
M. leprae recombinant proteins. *M. leprae* genes encoding candidate proteins derived from group VI (unknown function; ref Sanger database) were selected as described in detail previously [2]. The selected genes encoding the candidate proteins were amplified with PCR and cloned in pDEST17 expression vector containing an N-terminal histidine tag using the Gateway technology platform (Invitrogen, Carlsbad, CA). The recombinant proteins were overexpressed in *E. coli* BL21 (DE3) and purified from endotoxins. The endotoxin level in each recombinant protein was measured using a Limulus Amebocyte Lysate (LAL) assay and was below 50IU/mg (Cambrex, East Rutherford, NJ) [7]. All recombinant proteins were tested to exclude antigen non-specific T cell stimulation and cellular toxicity by measuring the IFN-γ level induced during 6 days incubation. Responses to medium and PHA were used as the negative and positive references in the assay. PBMC from *in vitro* PPD unresponsive, Mantoux skin test negative healthy Dutch donors recruited at the Leiden University Medical Center (LUMC) (with no prior contact with leprosy or TB patients) were used in the test.
The *M. leprae* proteins ML0126, ML0840, ML1601, ML1602, ML1603, ML1604, ML0573, ML0574, ML0575, and ML0576 were tested in Nepal and Ethiopia. Other *M. leprae* proteins: ML0121, ML0141, ML0188, ML1788, ML0369 and ML0927 only in Ethiopia. ML0121, ML0141, ML0188 were kindly provided by Dr. JS Spencer through the NIH/NIAID "Leprosy Research Support" Contract N01 AI-25469 from Colorado State University (these reagents are now available through the Biodefense and Emerging Infections Research Resources Repository listed at (http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

M. leprae whole cell sonicate. Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage (Colorado State University, Fort Collins, USA through the NIH/NIAID Leprosy Contract N01-AI-25469).

Synthetic peptides. Synthetic peptides overlapping the complete sequence of protein ML0576, ML1989, ML2283, ML2346 and ML2567, produced as 20-mers overlapping 10 amino acids, were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA).

Study subjects. The study was approved by the local ethics committees in Nepal (Approval nr. 93 24-08-2006) and Ethiopia (RDHE/163-71/2006). Written informed consent was obtained from each participant before sample collection. A total of 160 HIV-negative individuals were recruited: 50 BB/BL, 35 TT/BT leprosy patients, 22 healthy household contacts of BL/LL patients (HHC), 30 healthy individuals from the same endemic area (EC) and 23 smear positive, pulmonary tuberculosis patients (TB). Leprosy patients recruited were new cases or patients on treatment for less than 3 months with or without leprosy reactions. Clinical, bacteriological and histological examinations were performed to classify the leprosy patients according to Ridley and Jopling [18]. HHC were defined as adults living in the same house as a BL/LL index case for at least the preceding six months. Both HHC and EC were assessed for the absence of signs and symptoms of tuberculosis and leprosy. Staff members working in both leprosy centers were excluded as EC. TB patients had been on chemotherapy for at least 2 months to enable some recovery of T cell function.

Lymphocyte stimulation tests (LST). Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC (2 x 10^6 cells/ ml) were plated in triplicate cultures in 96-well round bottom plates (Costar Corporation, Cambridge, MA) in 200 µl/ well of Adoptive Immunotherapy medium (AIM-V, Invitrogen, Carlsbad, CA). Synthetic peptides, recombinant protein, *M. leprae* whole cell sonicate or PPD (purified protein derivative of *M. tuberculosis*, SSI, Copenhagen, Denmark) were added at final concentrations of 10 µg/ ml. As a positive control stimulus a final concentration of 1 µg/ ml phytoheamagglutinin (PHA; Sigma, St. Louis, MO) was used. After 6 days of culture at 37°C at 5% CO_2, 90% relative humidity, 75 µl supernatants were removed from each well and triplicates were pooled and frozen in aliquots at –20°C until further analysis.

Whole blood assays (WBA). Venous blood samples from 9 leprosy patients (BT and BL) and 8 EC were collected in heparinized tubes and used for 24 hours undiluted and for 6 days diluted WBA. For the 24 hours WBA, 450µl of blood per well was added in 48 well plate and 50µl of stimuli; *M. leprae* whole sonicate, ML0121, ML0141, ML0188 diluted in AIM-V medium with final concentration of 10µg/ml and PHA; 1µg/ml was added. Plates were incubated at 37°C at 5% CO_2, 90% relative humidity for 24 hours. For the 6 days WBA, blood samples were diluted 1:10 with AIM-V medium (serum free) and 900µl was added per well. The same stimuli with final concentration of 10µg/ml other than PHA (1µg/ml) were added in 100ul volume per well. Plates were incubated at 37°C in 5% CO_2 and 90% relative humidity for 6 days. IFN-γ ELISA was simultaneously done on supernatants of both assays.

IFN-γ ELISA. IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands). The cut-off value to define positive responses was set beforehand at 100 pg/ ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically < 20 pg/ ml. Lyophilized supernatant of PHA cultures of PBMC from an anonymous Buffy coat (LUMC, The Netherlands) was provided to both sites as a reference positive control supernatant.

TABLE 1
Study population at the two participating sites

Site	P[1]	Category	BI (mean)	Sex ratio[2]	Mean age (yr)
Anandaban (Nepal)	1.56	BB/BL	3.88	35/2	37.0
		BT/TT	0	18/7	36.9
		HHC	-[3]	14/6	32.7
		EC	-[3]	11/7	22.2
		TB	-[3]	9/5	29
Addis Ababa (Ethiopia)	0.60	BB/BL	1.64	9/4	29.2
		BT/TT	0.56	6/4	30.8
		HHC	-[3]	0/2	22
		EC	-[3]	4/8	27
		TB	-[3]	4/5	23

[1] prevalence per 10,000 individuals
[2] male/ female ratio
[3] not applicable

TABLE II
***M. leprae* specific candidate proteins**

Accession gene number*	Functional Classification	Protein product	Mr (kD)	Percentage identity** with *Mycobacterium*:								
				murine and human RT-PCR	*bovis AF2122/97 blastp*** (28)	*tuberculosis H37Rv Blastp*** (28)	*paratuberculosis K10 blastp*** (30)	*microti*# tblastn** (28)	*marinum*# tblastn** (28)	*avium*# tblastn ** (29	*ulcerans* # tblastn ** (31	*smegmatis MC2*# tblastn ** (29)
ML0126	VI	Hypothetical	31	+	<30%	<30%	<30%	<30%	<30%	<30%	<67%	<30%
ML0369	VI	Hypothetical	13	+	<30%	<30%	MAP4250c 38%	<30%	<33%	<39%	<37%	<33%
ML0573	VI	Hypothetical	10	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0574	VI	Hypothetical	11	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0575	VI	Hypothetical	7	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0576	VI	Hypothetical	8	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0840	VI	Hypothetical	48	+	<30%	<30%	MAP2122 59%	<30%	<30%	<64%	<30%	<47%
ML0927	VI	Hypothetical	11	+	<30%	<30%	MAP1963c 36%	<30%	<36%	<35%	<33%	<30%
ML1601	VI	Hypothetical	13	+	<30%	<30%	MAP3249 33%	<30%	<30%	<33%	<30%	<30%
ML1602	VI	Hypothetical	11	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1603	VI	Hypothetical	9	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1604	VI	Hypothetical	14	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1788	VI	Hypothetical	17	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%

Functional Classification VI: unknown; ** blast reports were run December 2004; blastp = protein vs. protein; tblastn = protein vs. translated DNA. # unfinished genomes. Ref: (13)

Results

Testing *M. leprae* proteins and peptides in different leprosy endemic sites harbouring variable genetic backgrounds is essential to develop diagnostic tools that can be widely applied. In this study, a total of 160 individuals (50 BB/BL, 35 TT/BT, 22 HHC, 30 EC and

23 TB) from two endemic sites Anandaban in Nepal and Addis Ababa in Ethiopia were enrolled (Table 1).

PBMC or whole blood samples from all individuals were stimulated for 6 days in LST or in 24 hours WBA with 21 *M. leprae* proteins and 10 peptide pools each consisting of overlapping 20-mer peptides covering the sequence of five *M. leprae* proteins (ML0576, ML1989, ML1990, ML2283 and ML2567) previously shown to be specific for *M. leprae* exposure in a Brazilian population [8;11]. The peptide pools were composed of a total of 50 peptides [ML0576 (n = 7), ML1989 (n = 11), ML1990 (n = 7), ML2283 (n = 10), ML2567 (n = 15). Each pool consisted of five peptides (Table 3). Samples with high IFN-γ values (> 200 pg/ml) in the unstimulated negative control wells and with low IFN-γ values (< 100 pg/ml) in response to PHA (positive control for the assay) were excluded from the analysis.

Differential recognition of antigens by individuals within test groups

M. leprae unique proteins ML0573, ML1601 and ML1604 induced IFN-γ responses in EC. These responses clearly showed two distinct subgroups where 50% of the group showed high response and 50% very low (Figure 1).The difference in IFN-γ response against the unique proteins among the endemic controls could be due to the difference in the level of exposure to *M. leprae* (Geluk *et al.,* manuscript in preparation).

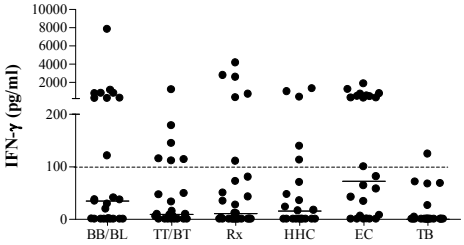
Patients with Type 1 leprosy reactions (RR) recognised ML0573 (48%) more frequently than ML1601 (26%) and ML1604 (30%). Although the median response was not above the cut-off for positive responses (i.e. 100 pg/ml), 40% of BB/BL patients also showed positive responses to ML0573 and ML1601 and 33% HHC and 35% TT/BT to ML1604 (Figure 1).

TABLE III

M. leprae peptide pool composition

Peptide pools	**pool 1 ML0576**	**pool 2 ML0576 & ML1989**	**pool 3 ML1989**	**pool 4 ML1989 & ML1990**	**pool 5 ML1990**
	5 6 7 8 9	10 45 *12 13 14*	*15 16 17 18 19*	*20 21 53* 22 23	24 25 26 27 28
Pool 6	*5*	10	*15*	*20*	24
Pool 7	*6*	45	*16*	*21*	25
Pool 8	*7*	*12*	*17*	*53*	26
Pool 9	*8*	*13*	*18*	22	27
Pool 10	*9*	*14*	*19*	23	28
	pool 11 ML2283	**pool 12 ML2283**	**pool 13 ML2567**	**pool 14 ML2567**	**pool 15 ML2567**
	29 30 31 32 33	*34 35 36 37 54*	*38 39 40 41 42*	*43 44 45 46 47*	*48 49 50 51 52*
Pool 16	*29*	*34*	*38*	*43*	*48*
Pool 17	*30*	*35*	*39*	*44*	*49*
Pool 18	*31*	*36*	*40*	*45*	*50*
Pool 19	*32*	*37*	*41*	*46*	*51*
Pool 20	*33*	*54*	*42*	*47*	*52*

ML0126



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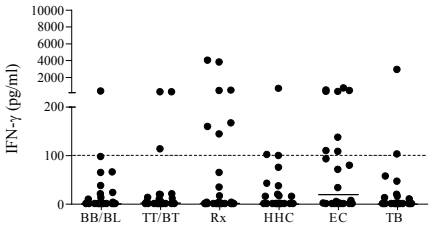

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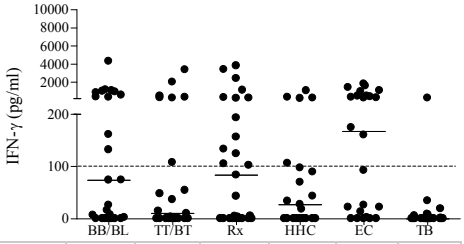
ML0573



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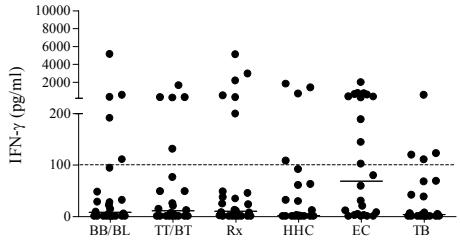
ML0574



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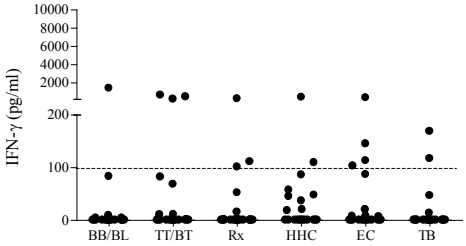
ML0575



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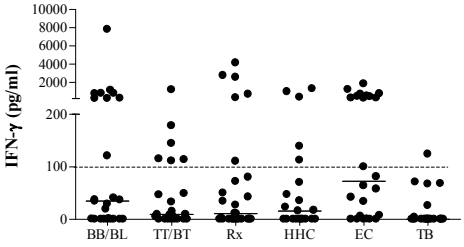

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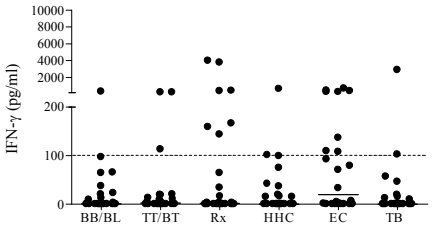

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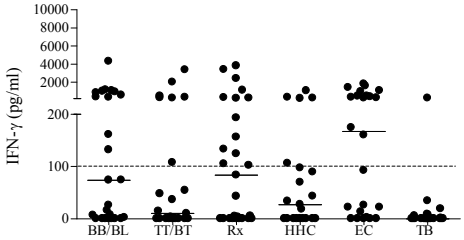
ML0573



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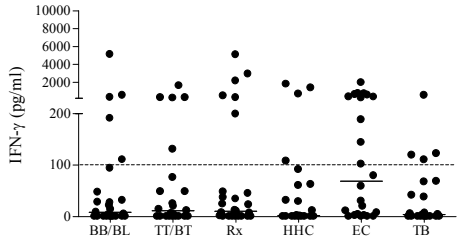
ML0574



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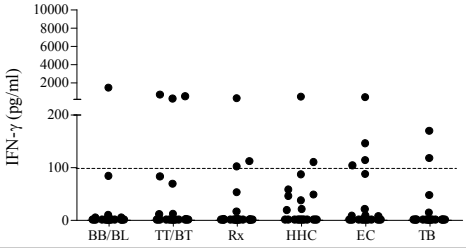

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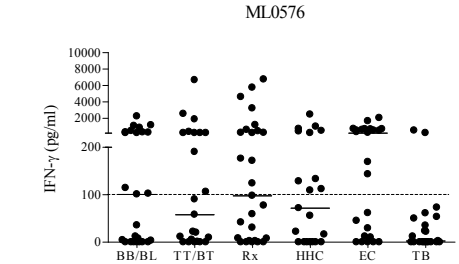

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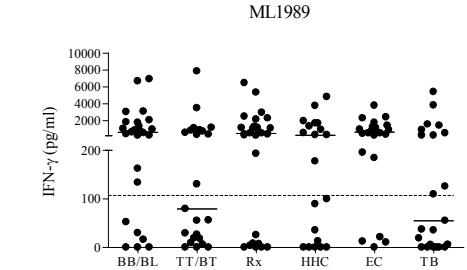
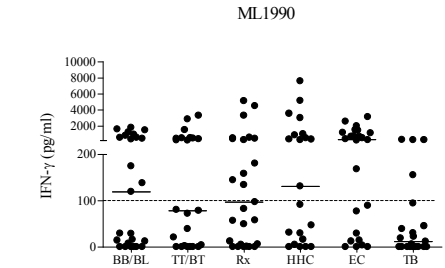
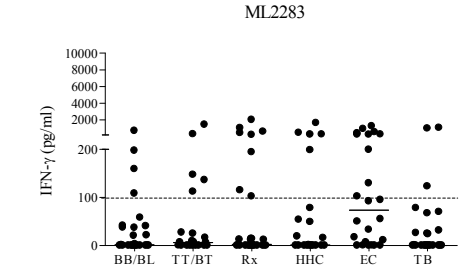
>200pg/ml	10/25	8/23	10/27	6/21	12/24	2/23



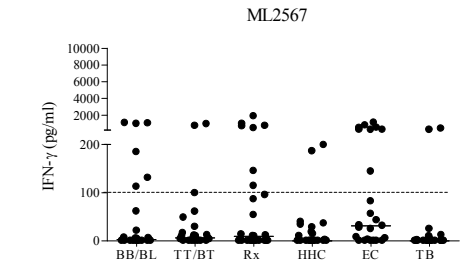
	BB/BL	TT/BT	Rx	HHC	EC	TB
<100pg/ml	6/25	12/23	8/27	9/21	4/24	12/23
100-200pg/ml	2/25	1/23	1/27	1/21	2/24	2/23
>200pg/ml	17/25	10/23	18/27	11/21	18/24	9/23



	BB/BL	TT/BT	Rx	HHC	EC	TB
<100pg/ml	12/25	13/23	14/27	10/21	9/24	20/23
100-200pg/ml	3/25	0/23	4/27	1/21	1/24	1/23
>200pg/ml	10/25	10/23	9/27	10/21	14/24	2/23



	BB/BL	TT/BT	Rx	HHC	EC	TB
<100pg/ml	14/25	16/23	14/27	16/21	11/24	21/23
100-200pg/ml	2/25	1/23	6/27	1/21	2/24	0/23
>200pg/ml	9/25	6/23	7/27	4/21	11/24	2/23



	BB/BL	TT/BT	Rx	HHC	EC	TB
<100pg/ml	19/25	21/23	20/27	19/21	16/24	21/23
100-200pg/ml	3/25	0/23	2/27	2/21	1/24	0/23
>200pg/ml	3/25	2/23	5/27	0/21	7/24	2/23

Figure 1: IFN-γ production corrected for medium values induced in response to 14 *M. leprae* proteins in 6 days incubation of PBMC from multibacillary leprosy patients (MB; *n* = 45; 20 with leprosy reaction), paucibacillary leprosy patients (PB; *n* = 30; 7 with leprosy reaction), healthy household contacts (HHC; *n* = 22), healthy endemic controls (EC; *n* = 22) and TB patients (TB; *n* = 23).

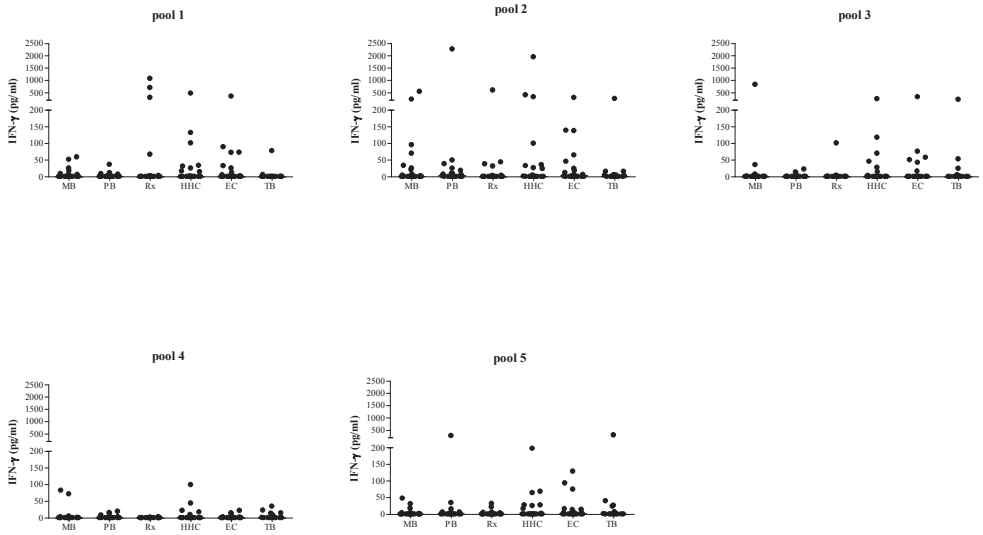
Similarly, ML0126 and ML0574 were more frequently recognised by EC (41%) than by leprosy patients but the median value was slightly below the cut-off. The responses in other groups were low for both proteins except the positive responses in 32% of BB/BL patients for ML0126 (Figure 1). Responses to ML1602 and ML1603 were low in all groups. However,

some individuals from BB/BL and EC group responded well to ML1603 but ML0840 and ML0575 were hardly detected in any of the groups (Figure 1).

Peptide pools induce only low IFN-γ responses in all test groups

The *M. leprae* proteins ML0576, ML1989, ML1990, ML2283 and ML2567 were analysed in five endemic countries previously and IFN-γ production was observed in response to these *M. leprae*-unique proteins in all test groups [9]. The current study analysed T cell reactivity in response to overlapping peptides of these proteins in LST. Peptide pools 1-5 and 11-15 (Table 3) were tested in the cohort from Nepal and peptide pools 6-10 and 16-20 were tested in Ethiopia (data not shown). The median IFN-γ levels in response to each peptide pool in all groups were found to be very low (Figure 2).

However, pool 1, 3, 5, 11, 14 and 15 containing peptides derived from ML0576, ML1989, ML1990, ML2283 and ML2567 respectively, showed some detectable responses in HHC and EC. Pool 2, composed of different peptides derived from ML0576 and ML1989 was recognised by few individuals from all groups except TB patients. Pool 12 and pool 13 contained peptides derived from ML2283 and ML2567 respectively, were recognised by some individuals from BB/BL, TT/BT, HHC and EC. Peptides from these antigens were reported previously also to be specifically recognised by *M. leprae* exposed (patients and HHC) in Brazil [11]. In general, responses induced by peptide pools were found to be low or absent. However, the low but detectable IFN-γ levels indicated the potential of these peptides for inducing specific T cell responses which may be enhanced by using different immunological boosting techniques such as addition of low concentration of cytokines or performing the test at temperatures above 37 °C related to fever [2;10].



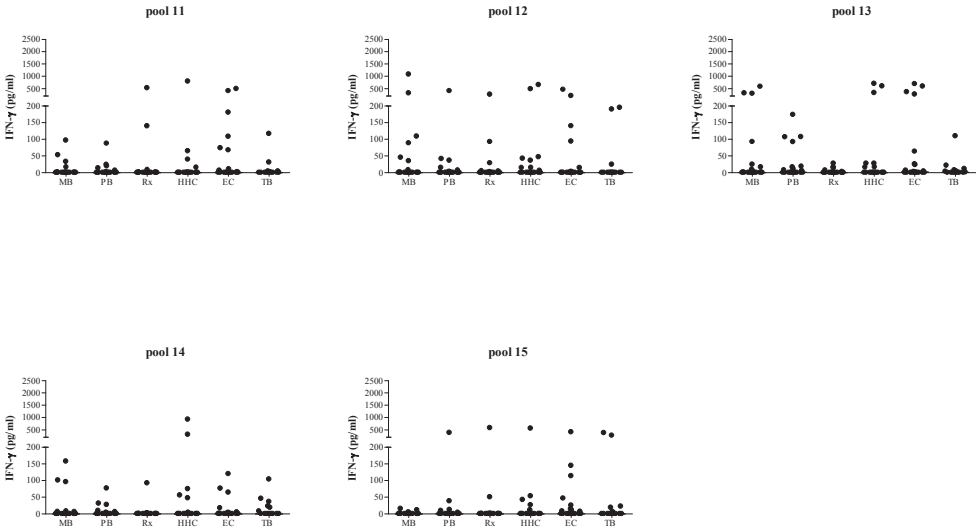


Figure 2: IFN-γ production corrected for medium values induced in response to 10 peptide pools in 6 days incubation of PBMC from BB/BL and TT/BT leprosy patients, healthy household contacts, healthy endemic controls and TB patients from Nepal.

Diluted WBA induced IFN-γ response in patients but not in EC

Finally, in order to compare what type of incubation would be preferable for *M. leprae*-specific IFN-γ detection in WBA, *M. leprae* proteins ML0121, ML0141 and ML0188 were simultaneously tested for their immunogenicity in an undiluted 24 hours WBA and a 1 in 10 diluted 6 days WBA (Figure 3).

The IFN-γ responses induced in whole blood samples derived from EC were significantly reduced (P=0.007 for ML0121 and P=0.049 for ML0141) in 6 days diluted WBA compared to the 24 hours undiluted WBA, whereas an increased IFN-γ response was seen in TT/BT patients in the 6 days diluted WBA.

Discussion

Since transmission of *M. leprae* infection is still ongoing as evidenced by the number of new cases in many endemic countries and the considerable number of patients presenting with Grade 2 disabilities, development of tools for early detection of *M. leprae* infection remains a key priority in combating leprosy [14;19].

The study described here represents the continuation of efforts to identify new *M. leprae* proteins and peptides that induce specific cellular immune responses and may be used in tools for early detection of infection in individuals living in leprosy endemic countries. The availability of whole genome sequences for *M. tuberculosis*, *M. leprae* and other mycobacterial species has tremendously supported this search effort through providing information on potential functions of proteins from unique genes. By selecting sequences that are uniquely found in *M. leprae,* we aimed to exclude T cell cross reactivity caused by homologous sequences. The *M. leprae* proteins tested in this study are hypothetical (categorised in group VI ref Sanger database) and their function is yet unknown except for ML1990, which was classified as a putative integral membrane protein. All proteins tested are unique to *M. leprae* except for ML1601, ML0369, ML0840 which have orthologues in *M.*

avium and ML0126 which has a homologue sequence (67 %) in *M. ulcerans*. Absence of responses in TB patients against the proteins confirmed absence of T cell cross reactivity with *M. tuberculosis*, also observed in Brazilian TB patients.

The IFN-γ responses against ML0573, ML1601 and ML1604 clearly divided the EC in two sub-groups: high and low responders. However, in the previous study, the responses from all Brazilian study groups were low for ML0573 and the responses to ML1604 were not specific as they were also recognised by individuals who did not respond to *M. leprae* whole sonicate *in vitro* [8] The aim of testing proteins in different endemic countries is to eventually design a widely applicable diagnostic tool. Therefore, using combinations of *M. leprae* proteins could be a method to overcome low and high responses in different leprosy endemic areas.

The high responses of EC described above could be due to exposure to *M. leprae* infection. However it is difficult to predict whether it is a sign of protection or infection leading to disease. Similar high responses were observed in EC in a previous study in response to other *M. leprae* proteins [9]. Further evaluation of these *M. leprae* proteins and screening of peptides from ML0573, ML1601 and ML1604 in healthy controls living in high, low and in non endemic areas and in close HHC in a longitudinal study will provide more insight on whether such responses are signs of protection against or susceptibility to disease. In addition, ML0573 may also be used in studies which aim to look for potential reaction predicting markers in leprosy patients as number of patients on reaction responded against this protein. In general, further evaluation of these potential proteins, their peptides and peptide pools in defined high risk groups could contribute in the development of early detection tools of leprosy infection.

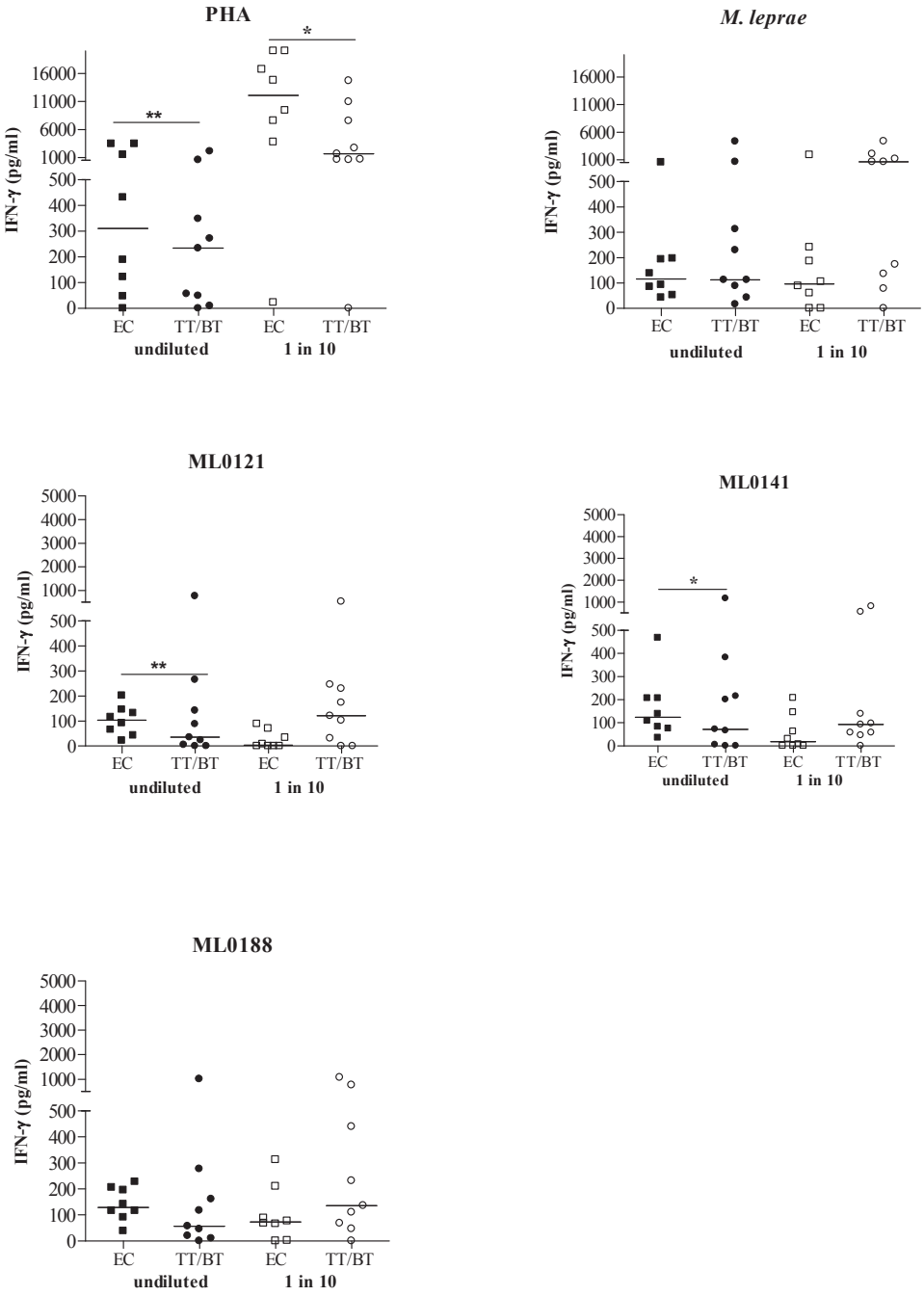


Figure 3: IFN-γ production in response to *M. leprae* recombinant proteins for EC (squares; $n = 8$) and BT/TT (circles; $n = 9$) in undiluted 24 hours WBA (undiluted; black symbols) or in 1:10 dilution 6 days WBA (1 in 10; open symbols). ** indicates P value = 0.007; * indicates P value = 0.05.

The IFN-γ levels produced in response to the peptide pools tested in this study in Nepal and Ethiopia were in general very low except for a few individuals with detectable responses (> 50 pg/ ml). Similarly, low responses against peptides were observed in previous studies [8;9]. However, considering those detectable responses against the peptide pools 2, 12 and 13, it could be useful to further screen single peptides from ML0576, ML2283 and ML2567 and also other potential proteins mentioned above especially in EC and HHC as was done previously in a Brazilian cohort [11]. As peptides are important in inducing specific responses, further boosting techniques should also be examined in order to enhance low but detectable IFN-γ responses [2;10]. Furthermore, identification of cytokines other than IFN-γ will also be necessary.

In determining what type of WBA would be beneficial to detect *M. leprae*-specific responses, we also compared diluted 6 days WBA to the undiluted 24 hour format. Interestingly, IFN-γ responses to *M. leprae* proteins in diluted 6 days WBA were significantly decreased for EC but not for leprosy patients, indicating reduction of some non-specific *ex vivo* responses observed in 24 hours WBA. Since the original aim of testing *M. leprae* proteins in WBA is to develop field-friendly diagnostic tools, 24 hours WBA would be preferred. However, diluted 6 days WBA is still very useful for selecting immunogenic *M. leprae* proteins which may induce specific memory T cell responses in *M. leprae* infected individuals. Therefore, working with diluted blood in 6 days WBA can be used in primary screening of proteins and peptides giving lower load and requiring small amount of blood [4].

The challenges in leprosy, prevention of deformities, disabilities, stigma, and interruption of transmission in leprosy endemic countries, require tools for early detection of *M. leprae* infection. Hence, identifying and screening candidate *M. leprae* proteins and peptides which could potentially induce cellular responses specific for *M. leprae* in individuals at high risk of developing leprosy should remain a continuous effort.

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References

1. Richardus JH, Habbema JD. The impact of leprosy control on the transmission of M. leprae: is elimination being attained? Lepr Rev 2007 Dec;78(4):330-7.
2. Global leprosy situation, 2010. Wkly Epidemiol Rec 2010 Aug 27;85(35):337-48.
3. Leprosy fact sheet (revised in February 2010). Wkly Epidemiol Rec 2009 Feb 5;85(6):46-8.
4. Meima A, Van Veen NH, Richardus JH. Future prevalence of WHO grade 2 impairment in relation to incidence trends in leprosy: an exploration. Trop Med Int Health 2008 Feb;13(2):241-6.
5. Dockrell HM, Brahmbhatt S, Robertson BD, Britton S, Fruth U, Gebre N, et al. Diagnostic assays for leprosy based on T-cell epitopes. Lepr Rev 2000 Dec;71 Suppl:S55-S58.
6. Dockrell HM, Brahmbhatt S, Robertson BD, Britton S, Fruth U, Gebre N, et al. A postgenomic approach to identification of Mycobacterium leprae-specific peptides as T-cell reagents. Infect Immun 2000 Oct;68(10):5846-55.
7. Leekassa R, Bizuneh E, Alem A. Prevalence of mental distress in the outpatient clinic of a specialized leprosy hospital. Addis Ababa, Ethiopia, 2002. Lepr Rev 2004 Dec;75(4):367-75.
8. Tsutsumi A, Izutsu T, kramul Islam MD, Amed JU, Nakahara S, Takagi F, et al. Depressive status of leprosy patients in Bangladesh: association with self-perception of stigma. Lepr Rev 2004 Mar;75(1):57-66.
9. Rafferty J. Curing the stigma of leprosy. Lepr Rev 2005 Jun; 76(2):119-26.
10. Cross H, Choudhary R. STEP: an intervention to address the issue of stigma related to leprosy in Southern Nepal. Lepr Rev 2005 Dec; 76(4):316-24.
11. Jacob JT, Franco-Paredes C. The stigmatization of leprosy in India and its impact on future approaches to elimination and control. PLoS Negl Trop Dis 2008;2(1):e113.
12. Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, Martins MV, et al. Identification of specific proteins and peptides in mycobacterium leprae suitable for the selective diagnosis of leprosy. J Immunol 2005 Dec 15;175(12):7930-8.
13. Geluk A, Klein MR, Franken KL, van Meijgaarden KE, Wieles B, Pereira KC, et al. Postgenomic approach to identify novel Mycobacterium leprae antigens with potential to improve immunodiagnosis of infection. Infect Immun 2005 Sep;73(9):5636-44.
14. Geluk A, van der PJ, Teles RO, Franken KL, Prins C, Drijfhout JW, et al. Rational combination of peptides derived from different Mycobacterium leprae proteins improves sensitivity for immunodiagnosis of M. leprae infection. Clin Vaccine Immunol 2008 Mar;15(3):522-33.
15. Geluk A, Spencer JS, Bobosha K, Pessolani MC, Pereira GM, Banu S, et al. From genome-based in silico predictions to ex vivo verification of leprosy diagnosis. Clin Vaccine Immunol 2009 Mar;16(3):352-9.
16. Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis 1966 Jul;34(3):255-73.
17. Oskam L, Slim E, Buhrer-Sekula S. Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Lepr Rev 2003 Sep;74(3):196-205.
18. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL. The continuing challenges of leprosy. Clin Microbiol Rev 2006 Apr;19(2):338-81.
19. Duthie MS, Goto W, Ireton GC, Reece ST, Cardoso LP, Martelli CM, et al. Use of protein antigens for early serological diagnosis of leprosy. Clin Vaccine Immunol 2007 Nov;14(11):1400-8.
20. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den HJ, Ottenhoff TH, et al. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein Expr Purif 2000 Feb;18(1):95-9.
21. Geluk A, van der Ploeg-van Schip JJ, van Meijgaarden KE, Commandeur S, Drijfhout JW, Benckhuijsen WE, et al. Enhancing sensitivity of detection of immune responses to Mycobacterium leprae peptides in whole-blood assays. Clin Vaccine Immunol 2010 Jun;17(6):993-1004.
22. Dockrell HM, Black GF, Weir RE, Fine PE. Whole blood assays for interferon-gamma: practicalities and potential for use as diagnostic tests in the field. Lepr Rev 2000 Dec;71 Suppl:S60-S62.